REMARKS

The last Office Action of April 2, 2009 has been carefully considered. Reconsideration of the instant application in view of the foregoing amendments and the following remarks is respectfully requested.

Claims 42-44 are pending in the application. No claims have been amended. A total of 3 claims is now on file. No amendment to the specification has been made. No fee is due.

It is noted that claims 42 and 44 is rejected under 35 U.S.C. §103(a) as being unpatentable over McCluskie in view of Wittig and Makkerh all of record.

It is further noted that claim 43 is rejected under 35 U.S.C. §103(a) as being unpatentable over McCluskie in view of Wittig and Liu of record.

REJECTION OF CLAIMS 42 AND 43 UNDER 35 U.S.C. §103(a) AS BEING UNPATENTABLE BY MCCLUSKIE IN VIEW OF WITTIG AND MAKKERH

At the outset, it noted that the McCluskie reference was already cited several years back in time and was then overcome by argument as the Examiner then cited the Schirmbeck reference a closer prior art. Since applicant has antedated the Schirmbeck reference, the Examiner now gives one more shot with McCluskie. However, it is evident from the record that the McCluskie reference is a weak reference and not relevant here.

The Examiner claims that the Wittig reference supplements McCluskie by teaching the dumbbell DNA expression construct and furthermore that Wittig teaches an NLS which "inherently comprises" the claimed PKKKRKV sequence. The Examiner does admit that Wittig does not teach the specific peptide, but claims that the specific NLS was known by Makkerh.

1. McCluskie is irrelevant here as already formerly discussed, due to starkly differing subject matter relative to the present invention.

The combination McCluskie/Wittig suffers from *ex post facto* analysis. If McCluskie is treated as the closest prior art, those skilled in the art would not refer to Wittig when using McCluskie as a starting point without knowing the outcome. Motivation to combine the two references is entirely lacking here.

2. The teaching of Wittig would produce various outcomes not leading to any chance of expected success.

3. McCluskie teaches away from the solution that Wittig teaches with respect to CpG immunostimulatory sequences.

1. McCluskie teaches a comparison of immune responses generated by different routes of plasmid administration in mice and non-human primates, plasmid expression vectors in a study focused on the **routes** of administration.

Hepatitis surface antigen expressing plasmids were delivered by means of 8 different methods of injections and 6 methods not involving injections. The findings in McCluskie demonstrate that the route of administration of plasmid DNA vaccines influence the strength and nature of the immune response in mice and non-human primates.

McCluskie is irrelevant prior art for the present invention for the following reasons. McCluskie only discloses plasmid expression constructs. As was known in the art at the time of the present invention, plasmid expression vectors demonstrated significant disadvantages due to unwanted toxic immunological side effects when administered as vaccines. Plasmid sequences may cause undesirable effects such as the production of antibodies against bacterial proteins expressed from cryptic eukaryotic expression signals, changes in the eukaryotic gene expression caused by the antibiotic resistance markers, and immune responses to CpG sequences. These disadvantages were known at the time of invention. Moreover, the present DNA construct lacks unmethylated immunostimulatory CpG motifs in the backbone of the DNA construct and thus distinguishes significantly from the prior art of McCluskie. In support of the safety

concerns in the plasmid based literature, applicant submits the Darquet publication from Gene Therapy. This publication is not considered prior art related to the subject matter but is cited to support the point made in relation to the drawback of plasmids.

2. McCluskie is concerned with an efficient method of plasmid injection. Improved administration and increased dosing of plasmid DNA are McCluskie's most important features. There is no suggestion of improving or modifying the structure and nature of DNA expression constructs. According to the recent *KSR* case, the Supreme Court did not negate the requirement for the TSM test under such circumstances; it just did not want the test to be applied rigidly. Here, clearly is a complete lack of teaching, suggestion or motivation to improve the DNA expression construct.

Being aware of the disadvantages of plasmid expression vectors, and the significant movement in the field of expression vector technology, one skilled in the art would not have looked to McCluskie for a modification of the vector structure. The invention rests on the object of eliciting a Th 1 immune response avoiding the controversial safety concerns of either plasmid or viral vectors (see also paragraph [0011] of the instant description).

The flaw of *ex post facto* analysis is that with the benefit of hindsight, certain developments seem possible that were not to be expected in a forward mode. Since McCluskie is not directed to the structural implication of vectors, no motivation or suggestion can be found in it to provide a better vector.

3. Significantly, Wittig and McCluskie provide opposing teachings. McCluskie teaches that the modification of a DNA expression vector may not be of importance in the vaccination of larger animals including humans (introduction, page 288, 2. and 3. paragraph). Moreover, any incentive to modify the plasmid technology as disclosed in McCluskie is an addition of CpG into the vector to improve immunostimulatory action. As stated in McCluskie on page 259: *"Considerable effort has been expended toward improving the efficacy of DNA vaccines through addition of immunostimulatory CpG motifs to plasmid vectors".*

Wittig on the other hand teaches an expression construct which strips away all unnecessary sequences from an expression vector including immunostimulatory CpG motifs. Therefore, the teaching and motivation in McCluskie is in direct opposition to the improvement which McCluskie is concerned with, thus teaching clearly away from the present invention.

4. The application of specific peptides was not obvious from the disclosure in Wittig. Wittig teaches that peptide chains can be covalently coupled to expression constructs in order o facilitate crossing of the endosomal membrane and nuclear localization (Wittig column 5). Wittig does not teach the specific peptide sequence but proposes three distinct peptides that could be added to DNA expression constructs: "the nuclear localization sequence from SV 40", the "signal peptide from HIV-gp41" and the "23N-terminal amino acids of haemaglutinine". If the peptide coupling as taught by Wittig were applied directly to McCluskie, various outcomes and thus uncertainty would arise, since it is not predictable which of the peptides would lead to success. Due to the significant difference in the three proposed peptides, and the uncertainty of applying any the three, the selection of the SV40 NLS is not a functional substitution, but rather an unobvious choice in accordance with the invention. In considering the unobviousness of the invention, it is irrelevant whether or not the exact peptide sequence was known in another context. The crucial selective step for the person skilled in the art is to decide from the three variants, which choice necessarily leads to uncertainty and likely success cannot be predicted from that.

It is also noted that in another context, the Examiner has taken the opposite position with regard to a sequence incorporating portions not claimed. Here the Examiner asserts that the peptide sequence "inherently" includes the prior art sequence. In that other context, the Examiner required a showing by applicant that extraneous amino acids in the sequence of the peptide have no function.

Accordingly, withdrawal of the rejection of claims 42 and 44 under 35 USC §103 (a) is respectfully requested.

REJECTION OF CLAIM 43 UNDER 35 U.S.C. §103(a) AS BEING UNPATENTABLE BY MCCLUSKIE IN VIEW OF WITTIG AND LIU

With respect to the combination McCluskie/Wittig, the foregoing remarks are applicable in the same manner and are incorporated herein by reference.

With respect to the Liu reference as teaching the specific sequence, applicant contends that whether or not the sequence is disclosed is irrelevant. In the context of the Examiner's rejection, the McCluskie reference must fall in view of applicant's arguments and since the main reference is inapplicable, the secondary reference thus also fails.

Accordingly, withdrawal of the rejection of claim 43 under 35 USC §103 (a) is respectfully requested.

In summary, the claimed invention is not obvious over McCluskie and Wittig in view of Makkerh or Liu since none of the references but specifically McCluskie does not teach the steps as claimed nor teach the DNA construct as claimed. Since the McCluskie reference must fall as a primary reference, the combination of McCluskie together with Wittig and Makkerh or Liu must also fall.

CONCLUSION

Applicant believes that when reconsidering the claims in the light of the above comments, the Examiner will agree that the invention is in no way properly met or anticipated or even suggested by any of the references however they are considered.

None of the references discloses a method as claimed to administer a vaccine of the type as set forth in claims 42 - 44.

In view of the above presented remarks and amendments, it is respectfully submitted that all claims on file should be considered patentably differentiated over the art and should be allowed.

Reconsideration and allowance of the present application are respectfully requested.

Should the Examiner consider necessary or desirable any formal changes anywhere in the specification, claims and/or drawing, then it is respectfully requested that such changes be made by Examiner's Amendment, if the Examiner feels this would facilitate passage of the case to issuance. If the Examiner feels that it might be helpful in advancing this case by calling the undersigned, applicant would greatly appreciate such a telephone interview.

Respectfully submitted,

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Gene Therapy (1999) 6, 209–218

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Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer

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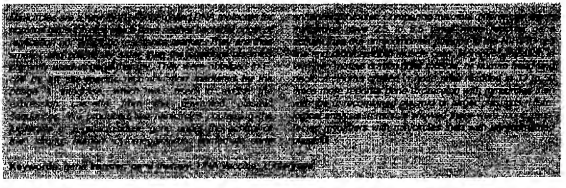

Introduction

Minicircles are new supercoiled DNA molecules for non-viral gene transfer, which have neither bacterial origin of replication nor antibiotic resistance gene.[1] Minicircles are obtained in *E. coli* by *att* site-specific recombination mediated by the phage λ integrase. Minicircles may contain no more than a eukaryotic expression cassette and the *attR* fragment resulting from the *attP/attB* recombination event. Thus they are a nondisseminating genetic material for nonviral gene therapy. Furthermore, minicircles carry only short bacterial sequences. Such sequences may cause undesirable effects such as the production of antibodies against bacterial proteins expressed from cryptic upstream eukaryotic expression signals,[2] changes in eukaryotic gene expression caused by the antibiotic resistance marker,[3] and immune responses to CpG sequences.[4–6]

In this study, we have produced new minicircles carrying the luciferase or β-galactosidase reporter gene under the control of the strong human cytomegalovirus immediate-early enhancer/promoter. The efficiency of gene transfer with these minicircles was compared with that of the corresponding unrecombined plasmid or larger plasmids, both *in vitro* in transformed primary cells, and *in vivo*, in muscle and experimental tumors. Our results suggest that the use of minicircles may be more efficient for *in vitro* and *in vivo* gene transfer than classical plasmids.

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Received 8 May 1998; accepted 16 September 1998

Results

Construction and production of CMV luc+ and CMV β-gal minicircles

The plasmids pXL3186 and pXL3187 contain the ColE1 origin of replication (ie they are derived from pBR322), a kanamycin-resistance gene, and, in the same orientation, the 385 bp *attP* site of λ phage and the 31 bp *E. coli* minimal *attB* sequence[7] (Figure 1). In pXL3186, the cDNA encoding the modified firefly luciferase was inserted between the λ *attP* and *attB* sites. In this 5.5 kb construct, the reporter gene is under the control of the CMV enhancer/promoter, fused to the herpes simplex virus thymidine kinase gene 5′ untranslated leader. The 5.5 kb construct also contains a polyadenylation signal from the bovine growth hormone gene. In pXL3187 the β-galactosidase gene was cloned between the λ *attP* and *attB* sites. In this 7.3 kb construct, the reporter gene is under the control of the CMV enhancer/promoter and has an SV40 polyadenylation signal.

Recombination was achieved by thermal induction of λ integrase at 42°C in *E. coli* D1210HP.[8] *E. coli* D1210, which lacks the thermosensitive lysogen, was used as a control. Phage λ integrase mediates site-specific recombination between the *attP* and *attB* sites. The two recombination sites are in the same orientation on the same replicon. Recombination results in the excision of a supercoiled minicircle carrying only the eukaryotic gene expression cassette and the recombinant *attR* site.[9] Int-

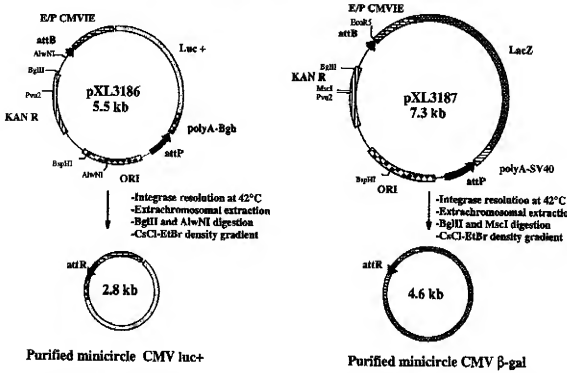


Figure 1 Plasmids pXL3186 and pXL3187: restriction map and scheme for minicircle production. The orientation of the two att sites of phage λ is indicated by arrows. Recombination between the att sites, catalyzed by λ integrase, which requires the E. coli proteins, IHF and FIS, resolves plasmid into two molecules: the miniplasmid, which contains the unwanted procaryotic sequences and the recombined attL site (not shown), and the minicircle, which contains the reporter gene and the recombined attR site. Ori, ColE1-derived origin of replication; Kan R, kanamycin-resistance gene; E/P CMVIE, immediate-early enhancer and promoter of cytomegalovirus; polyA-bGH, polyadenylation signal from the bovine growth hormone gene; polyA-SV40, polyadenylation signal from simian virus; Luc+, modified firefly luciferase gene; LacZ, β-galactosidase gene.

mediated recombination on pXL3186 produced a 2.8 kb minicircle with a CMV *luc*+ cassette, whereas a 4.6 kb minicircle containing a CMV β-galactosidase cassette is obtained after recombination on pXL3187 (Figure 1). In both cases, a 2.7 kb derivative plasmid containing the origin of replication, the antibiotic marker gene and the recombinant *attL* site was generated.

Extrachromosomal DNA from D1210HP pXL3186 and D1210HP pXL3187 cultures shifted from 30°C to 42°C was subjected to electrophoresis and all the expected bands were detected (Figure 2). There was no recombination in D1210 pXL3186 or D1210 pXL3187, indicating that the recombination was Int-mediated. There was unrecombined plasmid present in each case, showing that the reaction was not complete. Dimers and nicked form of each molecule were also visible.

Supercoiled molecules obtained by recombination were digested either with *Bgl*II and *Alw*NI (D1210HP pXL3186) or with *Bgl*II and *Msc*I (D1210HP pXL3187), which cut only the miniplasmid and the unrecombined plasmid, but not the corresponding minicircle (Figure 1). Supercoiled molecules were then isolated by CsCl ethidium bromide (CsCl-EtBr) density gradient centrifugation. Agarose gel electrophoresis showed that the minicircles obtained were not contaminated with miniplasmid or the starting plasmid (Figure 3). Similar results were obtained by Southern blot analysis (data not shown) using the following ^{32}P-labeled probes: (1) the 967 bp *Pvu*II–*Bsp*HI

Figure 2 In vivo recombination in E. coli producing the minicircle. Extrachromosomal DNA from: lane 1, uninduced D1210 pXL3187; lane 2, D1210 pXL3187 induced by heating to 42°C; lane 3, D1210HP pXL3187 after thermal shift to 42°C; lane 4, uninduced D1210HP pXL3187; lane 6, uninduced D1210 pXL3186; lane 7, D1210 pXL3186 induced by heating to 42°C; lane 8, D1210HP pXL3186 induced by heating to 42°C; lane 9, uninduced D1210HP pXL3186; lane 10, linear DNA ladder; lanes 5 and 11, supercoiled DNA ladder. Sizes (in kb) of the supercoiled DNA ladder are indicated on the right. Left arrows: supercoiled miniplasmid (A), supercoiled CMV luc+ minicircle (B), and supercoiled CMV β-gal minicircle (C).

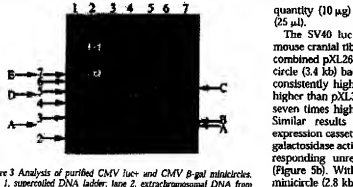


Figure 3 Analysis of purified CMV luc+ and CMV β-gal minicircles. Lane 1, supercoiled DNA ladder; lane 2, extrachromosomal DNA from D1210HP pXL3187 after thermal induction at 42°C; lane 3, uninduced D1210HP pXL3187; lane 4, supercoiled CMV β-gal minicircle obtained by MscI and BglII digestion and purification by CsCl-EtBr density gradient; lane 5, extrachromosomal DNA extracts from D1210HP pXL3186 after thermal induction at 42°C; lane 6, uninduced D1210HP pXL3186; lane 7, supercoiled CMV luc+ minicircle obtained by AhnVI and BglII digestion and purification by CsCl-EtBr density gradient. Sizes (in kb) of the supercoiled DNA ladder are indicated by arrows; (A) supercoiled miniplasmid; (B) supercoiled CMV luc+ minicircle; (C) supercoiled pXL3186; (D) supercoiled CMV β-gal minicircle; (E) supercoiled pXL3187.

fragment of pXL2776, which is specific for the miniplasmid or unrecombined plasmid backbone; (2) the 2.6 kb *StuI–EcoRI* fragment of pXL3072, which is specific for the CMV luc+ minicircle; and (3) the 4.3 kb *EcoRI–HindIII* fragment of pXL3187 which is specific for the CMV β-gal minicircle.

Minicircle dimers and relaxed minicircle copurified with the minicircle (Figure 3).

Comparison of in vitro transfection with minicircle and unrecombined plasmid

NIH3T3 fibroblasts were transfected using the cationic lipid RPR120535 complexed with either CMV luc+ pXL3186 or the derived CMV luc+ minicircle. For each cationic lipid:DNA ratio, luciferase expression was significantly higher with the minicircle than with the original plasmid (Figure 4a).

A similar result was obtained in NIH3T3 with the CMV β-gal minicircle (Figure 4c). The minicircle also gave significantly more expression when transfecting primary cells, such as rabbit aortic smooth muscle cells (Figure 4b and d). These *in vitro* experiments were reproduced between two and three times in triplicate in each cell line.

In vivo minicircle transfer

Reporter gene expression was studied after injection *in vivo* into either mouse cranial tibial muscle or experimental tumors obtained by implantation of human head and neck carcinoma TU182 cells into nude mice. *In vivo* gene transfer is a multifactorial process, depending on several parameters such as the number of expression cassettes, total amount of DNA and volume injected, as well as the presence of inflammatory contaminants. As a consequence, *in vivo* gene expression is rarely dependent in a linear fashion upon the quantity of reporter gene expression cassette administered. Therefore, in order to make a head to head comparison of different constructs as the tool for *in vivo* gene transfer, we injected the same quantity (10 μg) of each construct in the same volume (25 μl).

The SV40 luc expression cassette was injected into mouse cranial tibial muscle in a pXL3067 (15.3 kb), unrecombined pXL2650 (7.4 kb), pGL2 control (6 kb) or minicircle (3.4 kb) backbone. Luciferase gene expression was consistently higher with the minicircle, up to 32 times higher than pXL3067, 24 times higher than pXL2650 and seven times higher than with pGL2 control (Figure 5a). Similar results were obtained with a CMV β-gal expression cassette, where the minicircle (4.6 kb) gave β-galactosidase activity 13 times higher than that of the corresponding unrecombined plasmid pXL3187 (7.3 kb) (Figure 5b). With a CMV luc+ expression cassette, the minicircle (2.8 kb) gave luciferase activity up to 50 times higher than that of the unrecombined pXL3186 (5.5 kb) (Figure 5c). Gene expression was also dependent on the backbone used in the TU182 carcinoma system. For the SV40 expression cassette, the efficiency of gene transfer was 40 times higher with the minicircle than with the unrecombined plasmid pXL2650 and eight times higher with the minicircle than with the pGL2 control (Figure 6).

Comparative histological analysis of intramuscular gene transfer using the CMV luc+ minicircle and unrecombined pXL3186

Anterior tibial muscles injected with the CMV luc+ minicircle or pXL3186, as previously described, were used for a comparative histological study (Figure 7 and Table 1). Only myofibers expressed the transgene in anterior tibial muscle. They were located in small areas of the injected muscle, around the injection site. The intensity of luciferase staining differed greatly between transfected myofibers, from weak discrete labeling to strong diffuse labeling. This labeling was restricted to the sarcoplasm of the myofibers. The percentage of transfected myofibers per muscle was significantly higher for injection with CMV luc+ minicircle, with minimal levels of muscle damage, than for injection with pXL3186.

Discussion

Minicircles consist of a minimal DNA vector with no origin of replication or resistance marker. In this study, we have demonstrated that minicircles give higher levels of gene transfer *in vitro* and *in vivo* in the case of intramuscular and intratumoral transfer of naked DNA. We used several cell types and tissues and different reporter genes and promoters, demonstrating the potential of the minicircle as a DNA vehicle for nonviral gene transfer. Histological analysis showed that the percentage of transfected myofibers was markedly higher with the CMV luc+ minicircle than with the unrecombined plasmid pXL3186. The percentage of transfected myofibers, obtained with only 10 μg of the CMV luc + minicircle, was similar to that previously reported in an independent study with 50 μg of a standard CMV reporter gene construct.[10] The topology of transfected DNA has a major effect on gene transfer efficiency.[11] However, our preparations of DNA molecules, investigated by agarose gel electrophoresis, appeared to contain similar ratios of relaxed and supercoiled molecules, so DNA topology is thus unlikely to account for the differences in reporter gene expression observed. On the other hand, transgene expression has been shown to be affected by plasmid DNA contaminants

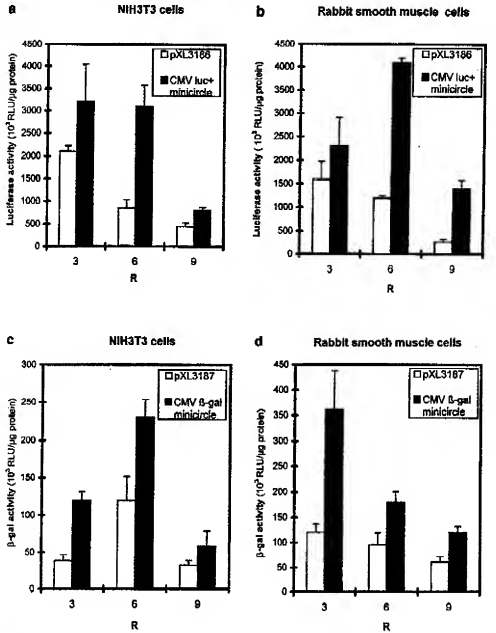


Figure 4 Reporter gene expression after in vitro transfection of NIH3T3 or rabbit smooth muscles cells with minicircle or unrecombined plasmid complexed with cationic lipid RPR 120535. Luciferase activity of the CMV luc+ minicircle and pXL3186 were compared using 1 µg pXL3186 per well, or 0.55 µg CMV luc+ minicircle with a complement to 1 µg with pBluescript KSII. The β-galactosidase activity of CMV β-gal minicircle and pXL3187 were compared using 1 µg pXL3187 or 0.63 µg of CMV β-gal minicircle with a complement of pBluescript KSII to 1 µg. Thus, the same molar concentration of luc+ cassette or β-galactosidase cassette and the same total amount of DNA were used for both minicircle and unrecombined plasmid. Transfections were performed at various ratios R of lipid (mol per µg DNA (R = 3, 6, 9). Results are given in RLU/µg protein for NIH3T3 (a and c) and rabbit smooth muscle cells (b and d). T bars indicate the standard deviation (s.d.)

such as lipopolysaccharide (LPS). In our protocol, minicircles could have increased level of purity, due to the fact that they were submitted to one more chloride–cesium protocol purification than parental plasmid. This could partly explain the increased expression observed with minicircle. However, we have verified that plasmid and minicircle preparation led to a similar low level of endotoxin. Finally, a complete assessment of the potential of the minicircle backbone over a classical plasmid backbone, requires, in the future, extensive studies at different

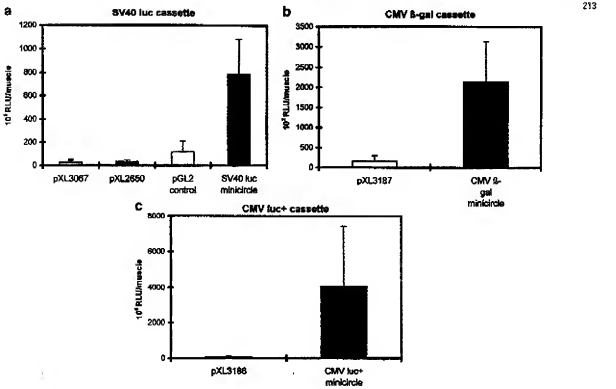


Figure 5 Reporter gene expression following intramuscular injection of minicircle or plasmids of various sizes and backbones. 10 µg DNA was injected into the cranial tibial muscle of C57BL6 mice (a and c) or transgenic lacZ mice (b). T bars indicate the standard error (s.e.) (n = 5–10).

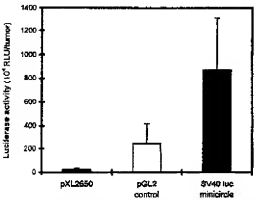


Figure 6 Reporter gene expression following intratumoral injection of SV 40 luc minicircle, pGL2 control or pXL2650. 10 µg naked DNA was injected into TU182 carcinomas on the flanks of nude mice. T bars indicate the s.e. (n = 11).

DNA dose and different mode of administration such as gene gun.

In vitro, the expression levels for vectors carrying the same reporter gene expression cassette suggest (especially in Figure 5a) an inverse relationship between the size of the DNA vector and the efficiency of reporter gene expression. Limited nonviral gene delivery *in vivo*, in terms of transfection efficiency, has been attributed to low bioavailability of the gene to the target cell.[12] DNA bioavailability involves: (1) diffusion of DNA into the tissue; (2) entry of DNA through the cell membrane: (3) DNA diffusion into the cytoplasm; and (4) DNA entry into the nucleus of quiescent post-mitotic cells. The first and third points are diffusion steps, which depend on the physicochemical properties of DNA, such as its diffusion coefficient, which is inversely proportional to its molecular weight.[13] Moreover, in muscles, the extracellular matrix, principally the epimysium and endomysium, act as a size exclusion barrier,[14] suggesting that the size of DNA molecules may have a major effect on the diffusion of plasmids into the myofibers.[15]

Recent studies seem to show that endocytosis is the major mechanism involved in the entry of DNA–cationic lipid complexes through the cell membrane *in vitro*.[16,17] This could also be the case for entry of naked DNA into muscle *in vivo*, after specific interactions between DNA and T-tubules or caveolae.[14,15] This step is limited by particle size: for example, liposomes larger than 200 nm in diameter are not efficiently taken up by the endocytic pathway (see for review Gao and Huang[18]).

Movement of DNA from the cytoplasm to the nucleus through the nuclear membrane is described as the more critical step for successful gene expression.[17] It has been shown that the rate of protein transport to the nucleus is influenced by the number of nuclear localization sequences in the protein, and most importantly, by the

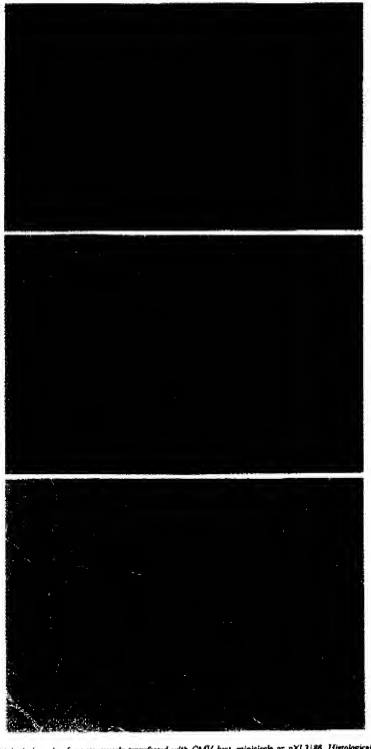


Figure 7 Comparative histological study of mouse muscle transfected with CMV hCK- minicircle or pX1.3186. Histological sections of cranial tibial muscles of C57BL6 mice, 1 week after injection of CMV hCK- minicircle (a, b and c) or of unrecombined pX1.3186 (d and e). Sections were stained for luciferase expression by immunohistochemistry (brown labeling) and counterstained with haematoxylin (purple labeling). (b and c) High magnification of (a). (e) High magnification of (d). Arrows indicate regenerated myofibers and inflammatory foci. Original magnification: (a) ×50; (d) ×100; (b and e) ×200; (c) ×400.

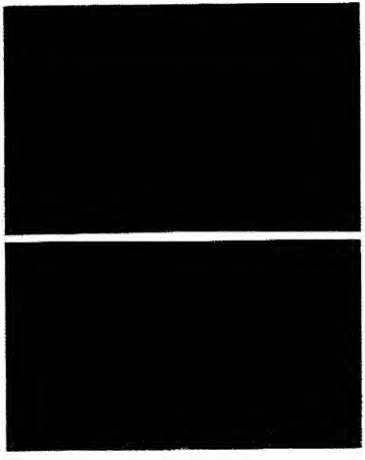
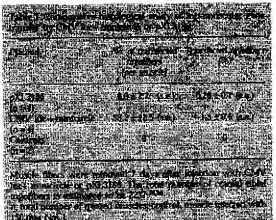


Figure 7 Continued.



size of the protein.[17,19,21] It is also conceivable that the size of transfected DNA molecules might determine their uptake through the nuclear pore into the nucleus.

All the above considerations may account for the higher levels of *in vivo* gene transfer obtained with the smaller minicircles, than with unrecombined plasmid and larger constructs. However, other factors may be responsible for higher efficiency of minicircles. In particular, the immunostimulatory potency of bacterial immunostimulatory sequences containing CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines might be important. These hexamers, which have been shown to affect gene expression by stimulating immunomodulatory cytokine[4-6] are 20 times more frequent in microbial than in mammalian genomes.[4] Such motifs are present more than a dozen times in the bacterial DNA backbone of pXL2776. Thus, the elimination of the bacterial DNA backbone in the minicircles might be partly responsible, by reducing inflammation and regeneration, for the higher levels of reporter gene expression in muscle. Finally, we cannot exclude a direct effect on transcription to explain the higher transgene expression observed with minicircles.

Since minicircles lack putative immunostimulatory sequences, they might be more suitable vectors for gene therapy than for DNA vaccines. On the other hand, their increased safety properties might be determinant for the extended use in large numbers of healthy subjects involved in vaccination.

The minicircles used in our experiments were purified

on density gradients which is not acceptable for clinical use. Wils *et al*[22] developed an affinity chomatography method for the purification of plasmid DNA, based on sequence-specific formation of a triple helix between immobilized oligonucleotides and a specific sequence present on the plasmid. This method could be used for large-scale production of minicircles. In conclusion, minicircles appear to be a promising tool for both *in vivo* and *in vitro* gene therapy strategies.

Materials and methods

Standard DNA manipulations

All standard manipulations, including digestion with restriction enzymes, purification of DNA fragments by electroelution, DNA electrophoresis, *E. coli* transformation, purification of supercoiled DNA by CsCl-EtBr density gradient, and Southern blot analysis were performed as previously described.[23] Intermediate constructions of plasmids were used to transform *E. coli* DH5α (F⁻, ϕ80lacZDM15, Δ(*lacZYA-argF*)*U169, deoR, recA1, endA1, hsdR17, phoA, supE44, λ-thi-1, gyrA96, relA1*) (Clontech Laboratories, Palo Alto, CA, USA). DNA concentration was measured by reverse phase HPLC analysis, using a Porus R2/11 column (100 × 4.6 mm; PerSeptive Biosystems, Cambridge, MA, USA) followed by UV absorbance at 280 nm, as previously described.[22]

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA), Gibco-BRL (Life Technologies, Cergy Pontoise, France) or Amersham (Amersham, Les Ulis, France).

Plasmid construction

pXL2776 was constructed from pXL2675, described by Wils and Ollivier in 1996 (patent reference FR 9603519): the minimal *attB* site[7] was inserted between the *HindIII* and SacI sites, and the *attP* site (position 27480 to 27860 in the published λ sequence of Sanger *et al*[24]) was inserted between the XbaI and NsiI sites. Thus, pXL2776 contained a multiple cloning site between the *attP* and *attB* sites in the same orientation, and a kanamycin expression cassette. It was used as a vector for further cloning.

pXL3072 consisted of the pUC19 backbone linearized by digestion with *PstI* and *XbaI*[25] into which the following fragments were inserted: (1) an *XbaI–HindIII* fragment containing the human cytomegalovirus enhancer/promoter (CMV) and the herpes simplex thymidine kinase ((tk) gene 5′ untranslated leader;[26] (2) a *HindIII–XbaI* fragment containing the cDNA encoding the modified firefly luciferase (luc+) from pGL3 Basic (Promega Corporation, Madison, WI, USA); and (3) a *PstI–XbaI* fragment containing the polyadenylation signal from the bovine growth hormone gene (PolyA bGH) produced by PCR from pCDNA3.1 (Invitrogen, Leek, The Netherlands).

The 2.6 kb StuI-CMVtk/luc+/PolyA bGH–EcoRI fragment of pXL3072 was inserted between the EcoRV and EcoRI sites of pXL2776, giving plasmid pXL3186 (Figure 1).

A plasmid was constructed from pCMVβ (Clontech Laboratories) by removing the *XhoI*–splice donor/splice acceptor–*SmaI* fragment, converting the 5′-overhang of *XhoI* to a blunt end with the Klenow polymerase[23] and religating the resultant DNA fragment. The 4.3 kb *EcoRI–β-gal–HindIII* fragment of this new plasmid was inserted between the *EcoRI* and *HindIII* sites of pXL2776, giving plasmid pXL3187. In this plasmid, the β-galactosidase (β-gal) gene was under the control of the immediate-early human CMV enhancer/promoter and a simian virus polyadenylation signal (Figure 1).

The SV40 luc minicircle (3.4 kb), plasmid pGL2 control (6 kb) (Promega Corporation), plasmid pXL2650 (7.4 kb) and plasmid pXL3067 (15.3 kb), which contain the same SV40-luc expression cassette, have been described.[1]

Production and purification of the CMV luc+ and CMV β-gal minicircles

Bacteria were grown at 30°C in LB medium containing 50 mg/ml kanamycin. Plasmids 3186 and 3187 were used to transform *E. coli* strains D1210 (F⁻ *hsdS20, supE44, ara-14, galK2, proA2, leuB6, rpsL20, xyl5, mtl1, recA, mcrB, Δ(mcrC-mrr), lacI*ᵠ)[27] and D1210HP (D1210 lysogenized with λ cI857 *xis*⁻ *kil*⁻ phage). The λ phage lytic cycle was initiated in D1210HP cultures by incubation for 10 min at 42°C, which caused site-specific recombination, leading to the production of a minicircle and a miniplasmid. The miniplasmid consisted mostly of the original plasmid with the reporter gene expression cassette deleted. Extrachromosomal DNA in these cells was purified using standard plasmid DNA purification techniques[23] with a CsCl-EtBr density gradient. The purified DNA was then digested with *MscI* and *BglII* (pXL3187) or with *AlwNI* and *BglII* (pXL3186). A second density gradient was used to separate the CMV luc+ minicircle or CMV β-gal minicircle from the linearized miniplasmid and from linearized pXL3186 or pXL3187 (Figure 1). Expression of plasmid DNA varies according to a large number of variables including differences between different preparations.[28] Thus, we had prepared different batches of plasmid and minicircle, which had been pooled in order to decrease the variability introduced by individual preparation.

CMV luc+ minicircle and CMV β-gal minicircle preparations were analyzed by Southern blotting. The 967 bp PvuII–BspHI fragment from pXL2776, the 2.6 kb StuI–EcoRI fragment of pXL3072, and the 4.3 kb EcoRI–HindIII fragment of pXL3187 were ^{32}P-labeled using the Rediprime kit (Amersham, Les Ulis, France).

In vitro gene transfer

Two cell lines were studied: NIH3T3 cells (murine fibroblasts: ATCC, Rockville, MD, USA) and rabbit primary aortic smooth muscle cells (RSM) obtained as previously described.[29]

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) containing 100 units/ml penicillin, 100 units/ml streptomycin, 20 mM L-glutamine, and 10% bovine serum (NIH3T3) or 20% fetal calf serum (RSM) (Gibco-BRL) at 37°C, in a 5% CO_2 humidified atmosphere. For transfection, confluent cells were treated with trypsin and used to seed 24-well microtiter plates at about 50 000 cells per well. Cells were transfected 18 h after seeding, at 60–80% confluence.

Equal volumes of the cationic lipid RPR120535 (formula: $[1_3N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CON HCH_2CON[(CH_2)_{17}\text{-}CH_3]_2]$)[30,31] diluted in water, and of supercoiled DNA diluted in 150 mM NaCl, were mixed and incubated for 10 min at 20°C. The total amount of DNA added to each well was adjusted to 1 μg with

pBluescript KS II (Stratagene Cloning System, La Jolla, CA, USA) for each well. Cells were washed twice with serum-free medium before transfection. Transfections were performed in 500 µl of serum-free medium by adding 50 µl of the lipid/DNA mixture to each well. Each transfection experiment was performed in triplicate. Two hours after transfection, serum was added to a final concentration of 10% for NIH3T3 and 20% for RSM cells. Luciferase or β-galactosidase activity was assayed 48 h later.

Cells were incubated for 30 min with lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2 diaminocyclohexane-*N, N, N', N'*-tetraacetic acid, 10% glycerol, and 1% Triton X-100) (200 µl per well). The cells were removed by scraping and the lysate centrifuged. Luciferase activity was determined using the Luciferase Assay System (Promega), and β-galactosidase activity was measured using the luminescent β-galactosidase Genetic Reporter System II according to the manufacturer's recommendations (Clontech Laboratories). A LUMAT LB 9501 luminometer was used (Berthold, Evry, France). Cumulative light emission was measured for 5 or 10 µl of supernatant over 10 s and was expressed in relative light units (RLU). The protein concentrations of the samples were determined using the Pierce BCA assay (Interchim, Asnières, France).[32] Results are expressed in RLU/µg protein ± standard deviation.

In vivo gene transfer

Wolff *et al*[33] showed that TE inhibits expression of genes transferred into muscle. Thus, we dialyzed the DNA used for transfection *in vivo* against 150 mM NaCl. We used the cranial tibial muscles of 6-week-old C57BL6 mice for experiments with pXL3186 and the CMV luc+ minicircle. Six-week-old transgenic *lacZ* mice[34] were used for experiments with pXL3187 and the CMV β-galactosidase minicircle. In these *lacZ* transgenic mice, the *lacZ* gene is under the control of a H 2 (a major histocompatibility complex class I gene) promoter. The fusion gene does not lead to expression in muscle.

For pXL2650, pCL2 control and the SV40 luc minicircle, all of which contain the SV40 luc expression cassette, we used the cranial tibial muscles of C57BL6 mice, or tumors induced by grafting TU182 cells in nude mice. TU182 cells (human head and neck squamous carcinoma cells) were a gift from Dr Richard J Cristiano (MD Anderson Cancer Center, Houston, TX, USA). Tumors were induced on the flanks of nude mice by subcutaneous injection of 10^7 TU182 cells in the exponential phase of growth. Tumors were detected by palpation after 2–3 weeks. They were injected with DNA constructs when they reached a volume of around 100 mm³. Mice were anesthetized by intraperitoneal injection with 0.25 ml of a mixed solution of ketamine (85.8 mg/kg) and xylazine (3.1 mg/kg) in 0.9% NaCl. Minicircle or plasmid DNA, which had been dialyzed against 150 mM NaCl, was injected into the cranial tibial muscle or the tumor, in a single injection of 25 µl, which contained 10 µg of DNA.

Animals were killed by cervical dislocation. Tumors were removed 2 days after intratumoral injection. Muscles were removed 7 days (β-galactosidase cassette) or 3 days (luciferase cassette) after intramuscular injection. The peak for luciferase expression in mice cranial tibial muscle occurred at the same time as reported in previous studies,[32] 3 days after injection (data not shown).

For evaluation of reporter gene activity by chemiluminescence, muscles or tumor nodules were placed into Eppendorf tubes with 750 µl lysis buffer containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Meylan, France) and minced finely with scissors. Samples were incubated for 30 min on dry ice, homogenized (Ultra-Thurax Diax600 Heidolph) for 1 min and centrifuged at 12 000 *g* for 5 min at 4°C. Luciferase activity and β-galactosidase activity were measured as described for *in vitro* transfection samples, using 10 µl of supernatant. The RLU were measured over 10 s. Results were normalized to total muscle or tumor volume.

Histological studies

The cranial tibial muscles of the mice were harvested 7 days after DNA injection, fixed for 4 h in 3.7% formalin in PBS, embedded in paraffin and 5 µm sections were cut. The paraffin was removed, sections were rehydrated and treated for immunohistochemistry. They were blocked in 10% bovine serum albumin for 30 min and incubated with a 1:500 dilution of polyclonal rabbit anti-mouse luciferase antibody (Europa Research Products/Cortex Biochem, San Leandro, CA, USA) for 1 h at 37°C. Sections were washed three times with PBS gelatin, and a 1:200 dilution of the secondary antibody biotinylated anti-rabbit IgG solution (Amersham) was added. The sections were incubated with a 1:400 dilution of avidin–biotin peroxidase complex (Amersham) and the reactions were detected with diaminobenzidine. All sections were counterstained in haematoxylin, dehydrated and mounted in Permount.

Acknowledgments

We thank G Pollet for reverse phase HPLC DNA analysis and K Berthelot for her help with RSM cell culture. We are grateful to G Byk for his generous gift of RPR120535. We thank J-F Mayaux and J-B Le Pecq for their constant interest and support during the work. This work was a part of the BioAvenir project supported by Rhône-Poulenc, the French Ministry of Research and the French Ministry of Industry.

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